Methods for Controlling IntraOcular Pressure

FIELD OF THE INVENTION

The present invention relates to the field of ophthalmology. In particular, the invention relates to the prevention and treatment of glaucoma and associated elevations of intraocular pressure, and to the treatment of ocular hypertension associated with other diseases or conditions.

REFERENCE TO RELATED APPLICATIONS

This application claims priority to US Provisional Application 60/133,180, filed May 7, 1999.

GOVERNMENT INTERESTS

This invention was supported in part by Grant Nos. EY08343 and EY01583 from the U.S. National Institutes of Health. The Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

The aqueous humor of the eye is formed by the ciliary epithelium, comprising two cell layers, whose apical membranes are juxtaposed. The outer pigmented ciliary epithelial (PE) cells face the stroma, while the inner nonpigmented ciliary epithelial (NPE) cells are in contact with the aqueous humor. Secretion involves primary solute transfer, primarily NaCl, with accompanying water movement, from the blood or supporting stroma, across the basolateral membranes of the PE cells into the aqueous humor in the contralateral posterior chamber of the eye (Cole, *Exp Eye Res* 25 (Suppl):161-176 (1977)). This provides an osmotic driving force for the secondary osmotic transfer of water down its chemical gradient, although a more direct coupling between water and solute may also proceed across the epithelia (Meinild *et* al., *J Physiol.* 508:15-21 (1998)).

The secretion of aqueous humor into the eye results as a consequence of two opposing physiological processes: fluid secretion into the eye by the NPE cells and fluid reabsorption (secretion out of the eye) by the PE cells. Thus, both release of chloride ions by the NPE cells into the adjacent aqueous humor enhance secretion,

and chloride ion release by the PE cells into the neighboring stroma reduces net secretion (Civan, *Current Topics in Membranes* 45:1-24 (1998)). Intraocular pressure reflects a balance between the rates of secretion and outflow of the aqueous humor. The aqueous humor leaves the eye in humans and primates primarily through the trabecular meshwork and canal of Schlemm, and in other mammals through the trabecular and angular aqueous plexus (Tripathi, In: The Eye, Chap. 3, pp 163-356, Davson & Graham (eds), Academic Press, New York, (1974)).

Glaucomas result from obstructed outflow from the aqueous humor, resulting in elevated intraocular pressure in the anterior chamber and visual loss attributed to progressive damage of the optic nerve, and consequent loss of retinal ganglion cells (Quigley *et al.*, *Invest. Ophthalmol. Vis. Sci.* 19:505 (1980)). Elevated intraocular pressure can also be caused by other conditions, such as impaired intraocular fluid transport caused by eye surgery, including surgery for glaucoma.

A major factor governing the rate of secretion is the rate of chloride ion (Cl) release from the NPE cells into the aqueous humor (Civan, *News Physiol. Sci.* 12:158-162 (1997)). Thus, the activity of the Cl channels is likely to be a rate-limiting factor in aqueous humor secretion, given the low baseline level of channel activity and the predominance of the chloride anion in the transferred fluid (Coca-Prados *et al.*, *Am J Physiol.* 268:C572-C579 (1995)).

Figure 1 depicts a minimalist, and necessarily incomplete, consensus model of aqueous humor secretion. (Carré *et al.*, *Curr Eye Res* 11:609-624 (1992); Chu *et al.*, *Invest Ophthalmol Vis Sci* 28:445-450 (1987); Wolosin *et al.*, *Exp Eye Res* 64:945-952 (1997)). As shown, NaCl is taken up from the stroma into the pigmented ciliary epithelial (PE) cells, supported by paired Na^+/H^+ and Cl^-/HCO_3^- antiports, and the Na^+-K^+-$2Cl^-$ symport. (Kaufman *et al.*, In: Textbook of Ophthalmology, Vol. 7, Podos & Yanoff (eds), Mosby, St Louis, pp 9.7-9.30 (1994); McLaughlin *et al.*, *Invest Ophthalmol Vis Sci* 39:1631-1641 (1998), Walker *et al.*, *Am J Physiol* 276:C1432-1438 (1999); Wiederholt *et al.*, In: Carbonic Anhydrase, Botré, Gross, Storey (eds), VCH, New York, pp 232-244 (1991). Edelman *et al.*, *Am J Physiol* 266:C1210-C1221 (1994), Wiederholt *et al.*, *Pflügers Arch* 407(Suppl 2):S112-S115 (1986)). It then diffuses through gap junctions into the inner nonpigmented ciliary epithelial (NPE) cell layer ((Coca-Prados *et al.*, *Curr Eye Res* 11:113-122 (1992); Edelman *et al.*,

1994; Mitchell *et al.*, *FASEB J* 11:A301 (1998); Oh *et al.*, *Invest Ophthalmol Vis Sci* 35:2509-2514 (1994); Raviola *et al.*, *Invest Ophthalmol Vis Sci* 17:958-981 (1978); Walker *et al.*, 1999; Wolosin *et al.*, In: The Eye's Aqueous Humor: From Secretion to Glaucoma, Civan (ed), Academic Press, Boston, pp 135-162 (1998)). Finally, it is released into the aqueous humor through the Na^+, K^+-exchange pump and Cl^- channels (Jacob *et al.*, *Am J Physiol* 271:C703-C720 (1996)).

The uptake step into the PE cells is largely electroneutral, although the underlying mechanism is not fully known. However, recent electron probe X-ray microanalyses (McLaughlin *et al.*, 1998) of excised intact rabbit iris-ciliary bodies, support the concept that the predominant uptake mechanism underlying baseline physiologic conditions is the pairing of the antiports. Indeed, the paired antiports can so elevate the intracellular Cl^- level as to favor the cellular release of NaCl through the Na^+-K^+-$2Cl^-$ symport. Yet, despite their putative importance of the paired Na^+/H^+ and Cl^-/HCO_3^- antiports, their molecular identity has remained a question.

Current treatment methods to relieve intraocular pressure include forming small laser penetrations in the eye to release excess pressure (*e.g.*, trabeculectomy), as well as the use of systemic and topical drugs for lowering intraocular pressure. At the present time, medical control of intraocular pressure and glaucoma consists of topical, oral or intravitreous administration of many compounds. See generally, Horlington, U.S. Pat. No. 4,425,346; Komuro *et al.*, U.S. Pat. No. 4,396,625; Gubin *et al.*, U.S. Pat. No. 5,017,579; Yamamori *et al.*, U.S. Pat. No. 4,396,625; Abelson, U.S. Pat. No. 4,981,871; and Bodor *et al.*, U.S. Pat. No. 4,158.005. Four primary classes of drugs are used: miotics (*e.g.*, pilocarpine, carbachol and acetylcholinesterase inhibitors); sympathomimetics (*e.g.*, epinephrine, dipivalylepinephrine and para-amino clonidine); beta-blockers (*e.g.*, betaxolol, levobunolol and timolol); and carbonic anhydrase inhibitors (*e.g.*, acetazolamide, methazolamide and ethoxzolamide). Another new type of drug, precursor prostaglandin compounds (*e.g.*, latanoprost), are also in current use.

To date, the most effective medical therapies are aimed at reducing intraocular pressure by inhibiting or reducing the net rate of aqueous humor formation (See generally, Shields, Textbook of Glaucoma, 3rd Ed., Williams & Wilkins, Baltimore (1992)). This can occur either by blocking unidirectional secretion from stroma to the

aqueous humor or by stimulating flow in the opposite direction (Caprioli *et al.*, *Yale J. Biol. Med.* 57:283-300 (1984); Civan *et al.*, *Exp. Eye Res.* 62, 627-640 (1996)). For example, miotics and sympathomimetics are believed to lower intraocular pressure by increasing the outflow of aqueous humor, while beta-blockers and carbonic anhydrase inhibitors are believed to operate by decreasing the formation of aqueous humor (Ritch *et al.*, (1996) In: The Glaucomas (eds Ritch, Shields, Krupin) 2nd ed. pp. 1507-1519, Mosby, St. Louis). The non-selective, topical, β- and β_1-adrenergic antagonists have proven to be useful for lowering the secretory rate of fluids in the eye (aqueous humor inflow), and thereby for controlling intraocular pressure (Gieser *et al.*, (1996). In: *The Glaucomas*, *supra*, pp. 1425-1448). For example, timolol reportedly binds to β-adrenergic receptors of the ciliary processes with high affinity (Vareilles *et al.*, *Invest. Ophthalmol. Vis. Sci.* 16:987-996 (1977)), and is among the most widely used and effective drugs for lowering the intraocular pressure of glaucomatous patients (Gieser *et al.*, 1996).

Nevertheless, each of the known drugs in current use is accompanied by significant adverse, systemic side-effects, even when administered topically, which may lead either to decreased patient compliance or to termination of therapy. Miotics tend to reduce the patient's visual acuity, particularly in the presence of lenticular opacities. Topical beta blockers, such as timolol, have been associated with side-effects such as fatigue, confusion, or asthma; while exacerbated cardiac symptoms have been reported after rapid withdrawal of topical beta blockers. Oral administration of carbonic anhydrase inhibitors, such as acetazolamide, while useful, have been associated with systemic side effects including chronic metabolic acidosis.

Unfortunately, because intraocular pressure, *e.g.*, related to glaucoma, progresses gradually and painlessly, it may not be detected until a late stage when irreversible damage to the optic nerve has already occurred. Accordingly, because of the insidious nature of glaucomas and other conditions affecting the intraocular pressure in the eye and the difficulties in treating them, there has been a long-felt need in the art for the development of methods for the safe and reliable prevention, control or treatment of elevated intraocular pressure, that can be taken before significant damage to the optical nerve occurs, and for the discovery of compositions that will cause fewer or reduced adverse side-effects when compared to present drugs.

Lower than normal intraocular pressure can also be problematic, caused for example, by a variety of conditions, such as surgery for glaucoma, retinal detachment, uveitis, and the like. However, since no drugs are presently available for the safe and effective prevention, modulation or regulation of reduced intraocular pressure without adverse side-effects, there remains a need for the development of drugs for the treatment of surgically-induced low or depressed intraocular pressure, as well as elevated intraocular pressure.

SUMMARY OF THE INVENTION

The present invention, therefore, meets a particular need in the art by providing a method for modulating or regulating intraocular pressure, in particular for preventing, treating or reducing elevated intraocular pressure. Using continuously cultured PE cells, the present invention provides characterization of the sodium/proton exchanger (antiport) which functions together with the chloride/bicarbonate exchanger (also an antiport) in the critical first step of the secretion of the aqueous humor, wherein fluids and salts are taken up from the stroma or body into the pigmented ciliary epithelial (PE) cell layer. In particular, the sodium/proton exchanger has now, for the first time, been identified as the NHE-1 member of the family of sodium/proton exchangers.

This discovery is particularly relevant because of the known sensitivity of the exchanger to a number of drugs, which are effective at very low concentrations. Consequently, in accordance with the present invention, control of the exchanger permits control or regulation of the secretion of the aqueous humor, permitting the prevention or modulation of the fluid in the intraocular space. Specifically, the present invention provides methods by which intraocular fluid pressure can be selectively and reversably increased, decreased, or maintained at a predetermined level, although primarily the invention will be useful to relieve or prevent elevated levels of intraocular fluid in, for example, glaucoma patients, before vision is adversely and permanently affected. In addition, low dosages permit the drugs to be used without any, or with minimal adverse side-effects.

The present invention provides a method for regulating, controlling or modulating aqueous humor secretion, comprising the step of administering to ciliary

epithelial cells of the aqueous humor, an effective secretion-modulating amount of a pharmaceutical composition comprising a modulator of one or more antiports. In one embodiment of the invention, the antiport is either a Na^+/H^+ exchanger or a Cl^-/HCO_3^- exchanger. In another embodiment, modulator is administered to more than one antiport, which comprise a Na^+/H^+ exchanger and a Cl^-/HCO_3^- exchanger.

Further provided is a method for regulating, controlling or modulating fluid pressure or intraocular pressure of the aqueous humor, comprising the step of administering to ciliary epithelial cells of the aqueous humor, an effective fluid pressure- or intraocular pressure-modulating amount of a pharmaceutical composition comprising a modulator of one or more antiports. In one embodiment of the invention, the antiport is either a Na^+/H^+ exchanger or a Cl^-/HCO_3^- exchanger. In another embodiment, modulator is administered to more than one antiport, which comprise a Na^+/H^+ exchanger and a Cl^-/HCO_3^- exchanger.

Also provided are methods wherein the Na^+/H^+ exchange occurs at the NHE-1 antiport, and wherein the Cl^-/HCO_3^- exchange occurs at the AE2 antiport.

In any of the preceding embodiments, the secretion in the aqueous humor cells is elevated, or the fluid pressure or intraocular pressure and wherein the modulator is administered in an amount, sufficient to reduce the elevated secretion. Moreover, the modulating effect is reversible upon cessation of administration of the modulator.

In addition, methods are provided wherein the modulator is administered to the cells *in vitro* or *in vivo*. The later methods offer regulation, control or modulation of fluid pressure or intraocular pressure in an individual.

Methods are provided wherein the Na^+/H^+ exchanger comprises NHE-1, and wherein the Cl^-/HCO_3^- exchanger comprises AE2. In light of the invention, it is clear that the paired NHE-1 Na^+/H^+ and AE2 Cl^-/HCO_3^- antiports are important components in the initial step in aqueous humor formation. Modulators of the antiports are beta blockers, *e.g.*, as timilol, amiloride analogs, *e.g.*, amiloride or ethyl-isopropyl-amiloride, and other compounds, *e.g.*, cariporide, at concentrations characteristic of the NHE-1 isoform.

In addition, methods are provided wherein an anion is transferred into the ciliary epithelial cells of the aqueous humor to block native chloride channels, a preferred embodiment of which is the transfer of cyclamate.

The invention will be more fully understood from the following detailed description of preferred embodiments, drawings and examples, all of which are intended to be for illustrative purposes only, and not intended in any way to limit the invention.

BRIEF DESCRIPTION OF THE FIGURES

In the following Figures, and in the Examples from which they are derived, values are presented as the means ±1 SE. The number of experiments is indicated by the symbol n or N.

Figure 1 depicts a minimalist model of NaCl secretion by the ciliary epithelium.

In this Figure 2 and in Figures 3 which follow, medians are indicated by the central horizontal lines, the lower and upper lines include all data between the 25th and 75th percentiles, and the 'whiskers' display the data range between the 10th and 90th percentiles. Circles are individual data points that lie outside of this range. The open and filled symbols present control and experimental results, respectively.

Figure 2 graphically depicts the effects of timolol on ciliary epithelial Na/P, Cl/P or K/P ratios in HCO_3^- -free or HCO_3^- solutions. Stars indicate significant differences from controls. (* = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

Figure 3 depicts a time course of the effects of timolol on ciliary epithelial Na/P, Cl/P or K/P ratios in HCO_3^- -solution. Stars indicate significant differences from controls. (* = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

Figure 4 depicts the effects of timolol and/or cAMP on ciliary epithelial Na/P, Cl/P or K/P ratios in HCO_3^- solution. The symbols represent the following, reading from the left: white box = control results; gray box = +timolol; hatched-line filled box = + cAMP; and black box = (+cAMP and timolol). Stars (*) indicate significant differences from the controls (* = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

Figure 5 depicts the effects of timolol and/or acetazolamide on ciliary epithelial Na/P, Cl/P or K/P ratios in HCO_3^- solution. The symbols represent the following, reading from the left of each set: white box = control conditions, hatched-line box = + acetazolamide, gray box = +timolol and black box = +acetazolamde and timolol. Stars (*) indicate significant differences from controls (* = $P<0.05$, ** =

P<0.01, *** = P<0.001).

Figure 6 depicts the effects of dimethylamiloride (50 μM) on ciliary epithelial Na/P, Cl/P or K/P ratios in HCO_3^- solution. The symbols represent the following: white box = control results; hatched-line filled box = experimental results. Stars (*) indicate significant differences from controls (* = P<0.05, ** = P<0.01, *** = P<0.001).

Figure 7 graphically depicts the voltage-dependent change in current produced by the seletive A^3-subtype adenosine agonist (IB-MECA), when most of the external chloride has been replaced by either aspartate (filled circles) or cyclamate (filled triangles). These currents are much smaller than those resulting from the presence of chloride (compare with Figure 8).

Figure 8 graphically depicts the measured current carried by chloride ions in the presence of an activator (IB-MECA) of the adenosine receptor as a function of voltage (in mV) applied across the cell membranes of immortalized cultured NPE cells.

Figure 9 graphically depicts the concentration-response relationship for $^{22}Na^+$ uptake by bovine PE cells in the presence of increasing concentrations of three inhibitors of Na^+/H^+ antiport activity: EIPA, cariporide and amiloride. In this and all subsequent figures, the error bars present ±1 SE.

Figures 10A and 10B graphically depict intracellular pH (pHi) response of acid preloaded PE cells to Na^+. After prealkalinization with NH_3/NH_4^+, the cells were abruptly acidified by superfusion with choline Cl solution at t = 3 minutes (in Figure 10A), and at t = 2 minutes (in Figure 10B). Figure 10A depicts the mean alkalinizing recovery in cells, which appeared after a delay of ~4 min after adding Na^+. Figure 10B depicts that the Na^+-triggered pHi recovery was entirely blocked by adding 3μM EIPA, and at the conclusion of the experiment, adding NH_3/NH_4^+ realkalinized the cells.

Figures 11A, 11B and 11C graphically depict the effect of brief trypsinization on the response to cellular acidification. Figure 11A depicts the mean pHi of control cells that were not exposed to trypsin; while Figure 11B depicts the data obtained following brief pretrypsinization. Figure 11C presents the two sets of data on the

same time scale to emphasize the faster response produced by reducing the area of attachment of the cells to the culture dish.

Figure 12 graphically depicts the effect of Cl^- and Na^+ removal on intracellular pH in the PE cells in the presence of HCO_3^-. Replacement of Cl^- by gluconate produced mean alkalinization, whereas return of Cl^- to the cells triggered a return to pHi

Figure 13 graphically depicts the effect of DIDS on Cl^-/HCO_3^- exchange in PE cells.

Figure 14A and Figure 14B graphically depict the effects of DIDS and trypsin on the PE cellular response to external Cl^- removal. Figure 14A presents the mean results obtained after cells were exposed to trypsin for 5min; whereas Figure 14B displays the averaged data from comparable cells in another dish studied on the same day, but without exposure to trypsin. The response to a second removal of Cl^- in Figure 14B was blunted by the addition of 100μM DIDS in the perfusate.

Figure 15 graphically depicts the baseline volume regulatory responses of bovine PE cells, as shown over a 50 minute period of observation, at 34°C. In Figures 15-18, the insets depict the regulatory volume increase (RVI) under control and experimental conditions at higher sensitivity and with the initial points aligned initially at the same relative volume (at t=28min).

Figure 16 graphically depicts the effect of dimethylamiloride on the RVI.

Figure 17 graphically depicts the effect of bumetanide on the RVI in the presence and absence of HCO_3^- (N=4).

Figure 18A, 18B and 18C graphically depict the effect of DIDS and bumetanide on the RVI. Neither 10 μM bumetanide (Figure 18A, N=9), nor 500 μM DIDS (Figure 18B, N=3) inhibited the volume recovery, but the two inhibitors together blocked the RVI (Figure 18C, N=8, $P<0.05$).

Figure 19 depicts the separation of products of RT-PCR amplification of AE anion exchanger transcripts from human ciliary process, separated on a 1% agarose gel. Expected migration positions (AE1, 754 bp; AE2, 368 bp; cAE3 982 bp; and bAE3, 891 bp) are indicated at right of the gel. cDNA loads derive from the following equivalent amounts of reverse transcribed total RNA: 12.5 and 17.5 ng for all lanes 1 and 2; 50 ng for AE1 lanes 4 and 5; 10 ng for AE2, cAE3, and bAE3 lanes

4 and 5. RNA was from human ciliary body (lanes 1 and 2); water control (lane 3), human heart (lane 4) and 293 human embryonic kidney cells (lane 5).

Figure 20 depicts the immunocytochemical detection of AE2 polypeptide. Figure 20A depicts the immunostaining of bovine PE cells with labeled antibody to the conserved mouse AE2 C-terminal peptide (residues 1224-1237). As shown in Figure 20B, this staining was abolished by the addition of AE2 peptide antigen, but the immunostaining was nearly completely retained in the presence of an excess of the corresponding AE3 C-terminal peptide antigen (Figure 20C), supporting the specificity of AE2 immunostaining in the PE cells. Bar, 25 μm.

DETAILED DESCRIPTION OF THE INVENTION

The methods and compositions of the present invention are intended for treatment of glaucoma and other conditions, which manifest elevated intraocular pressure in the eye of a patient, particularly human patients, but also including other mammalian hosts. Glaucoma is a term which embraces a group of ocular diseases characterized by elevated intraocular pressure levels which can damage the eye, and destroy the optic nerve and related ganglia. In addition, normotensive glaucoma is characterized by what would appear to be a nonelevated intraocular pressure. However, for the patient suffering from normotensive glaucoma, the apparently normal pressure is sufficiently high for that particular patient as to cause the same types of nerve and vision damage as elevated pressure would cause in patients with other glaucomas. Therefore, the glaucomas treated by the methods of the present invention are not limited exclusively to elevated intraocular pressure. Other conditions which result in elevated intraocular pressure levels include cataract surgery, steroid treatment, and treatment with other drugs known to cause intraocular pressure. The methods and compositions of the present invention are intended to treat all such conditions, preferably to lower the intraocular pressure to a manageable and safe level. Moreover, the methods are also effective in the treatment of lower than normal intraocular pressure levels.

The present invention provides a cultured bovine pigmented ciliary epithelial cell model, which when used as a representative model of those in the human eye, display: (1) pharmacologically distinctive Na^+/H^+ exchange demonstrating an NHE-1

antiport. (2) Na^+-independent Cl^-/HCO_3^- exchange, and (3) a regulatory volume increase involving the participation of both exchangers. It also provides (4) an AE2 epitope, and in addition (5) RT-PCR detected expression of AE2 mRNA in human ciliary body, but not of AE1, bAE3 or cAE3. Therefore, a specific AE anion exchanger has now been identified and characterized as part of the present invention, which controls the first stage of secretion of the aqueous humor, permitting for the first time the controlled regulation of the secretion. Consequently, since a number of drugs are known to affect the AE anion exchanger, it is now possible to control the fluid, salt or solute levels in the aqueous humor.

Medical therapy of glaucoma commonly aims at slowing aqueous humor formation by the ocular ciliary epithelial bilayer, but prior to the present findings, the underlying mechanisms were poorly understood. Current drugs prescribed for glaucoma, in the form of eyedrops, include pilocarpine, timolol, betaxolol, levobunolol, metipranolol, epinephrine, dipivefrin, latanoprost, carbachol, and potent cholinesterase inhibitors such as echothiophate and carbonic anhydrase inhibitors such as dorzolamidet. Many of these effective approaches to medical therapy of glaucoma involve a reduction in the rate of flow of fluids into the eye. However, none of these drugs are satisfactory, in part due to adverse side effects and inconvenient dosing schedules.

The present invention provides new understanding of the sodium/proton exchanger, and its functional relationship with the chloride/bicarbonate exchanger (the "antiports"), regarding the uptake of salts from the body into the PE cells. More particularly, identifying and characterizing a Na^+/proton exchanger as the antiport, permits strategies to be developed to use drugs at very low, focussed concentrations for preventing, modulating or regulating intraocular pressure, most particularly for treating or reducing elevated intraocular pressure.

Without being limited to a particular theory, the process appears to proceed in three steps. The first step in secretion is NaCl uptake from the stroma into the pigmented ciliary epithelial (PE) cell layer by electroneutral transporters. The second step involves the movement of salts and water from the PE cells across the gap junctions into the nonpigmented ciliary epithelial (NPE) cell layer abutting the

aqueous humor. The third and final step is the release of fluids and salts or other solutes into the aqueous humor by the contiguous NPE cells.

Previous reports by the inventors have indicated that the sodium/proton exchanger (or "antiport") is very important in the first step, including the uptake of fluids and salts into the PE cells. However, the mechanics and identity of the exchanger were unknown, and not characterized. By comparison, the present invention demonstrates that both paired Na^+/H^+ and Cl^-/HCO_3^- antiports and the Na^+-K^+-$2Cl^-$ symport are involved in net uptake.

In the normal PE / NPE cell bilayer, water and small non-polar molecules would typically cross rapidly. However, charged molecules and salts cross the cell barrier through carrier transmembrane proteins. Some carrier proteins ("uniports") simply transport a single solute from one side of the cell layer to the other. Others function as coupled transporters, in which the transfer of one solute depends upon the simultaneous or sequential transfer of a second solute, either in the same direction (a "symport"), or in the opposite direction (an "antiport"). Many active transport systems are driven by the energy stored in ion gradients, some of which function as symports, others as antiports. Two important examples of ion gradients used to drive an antiport system are the antiports that function together to regulate intracellular pH in many animals.

Almost all vertebrate cells have a NA^+ driven antiport, called an Na^+-H^+ exchange carrier or "exchanger," which plays a crucial role in maintaining intracellular pH ("pHi," usually around 7.1 or 7.2). This carrier couples the efflux of H^+ to the influx of Na^+, and thereby removes excess H^+ ions produced as a result of the acid-forming reactions in the cell. Thus, the Na^+-H^+ exchanger regulates pHi. At higher pHi, the exchanger is inactive, but activity increases as the pHi becomes more acid and approaches 7.4.

The Cl^--HCO_3 exchanger, like the Na^+-H^+ exchanger, regulates pHi, but in the opposite direction. Its activity increases as pHi rises, increasing the rate at which HCO_3^- (also referred to as bicarbonate) is ejected from the cell in exchange for Cl^-, thereby decreasing pHi. Flow through the exchangers is driven by the electrochemical gradient for the ion.

Elevated intraocular pressures often exceed 20 mmHg and it is desirable that such elevated pressures be lowered to below 18 mmHg. In the case of low-tension glaucoma, it is desirable for the intraocular pressure to be lowered below that exhibited by the patient prior to treatment. Intraocular pressure can be measured by conventional tonometry techniques.

The methods and compositions of the present invention are also intended for treatment of hypotonia and/or reduced intraocular pressure conditions of the eye. Reduced intraocular pressures are generally considered below about 8 mmHg. Such conditions may result from a variety of causes, such as surgery for glaucoma, retinal detachment, uveitis, and the like.

Using continuously cultured bovine PE cells as a widely-accepted, representative model for the human ciliary epithelial cells, the present invention demonstrates that acid-activated $^{22}Na^{-}$ uptake is inhibited by a variety of drugs. The exemplified inhibitors described in detail in the Examples include cariporide, EIPA (ethyl-isopropyl-amiloride) and amiloride, at concentrations characteristic of the NHE-1 isoform (The term NHE is an abbreviation in which N refers to sodium, H to proton, and E to exchanger).

Nevertheless, applicable compounds would include any of the beta blockers (including topical, β- and β_1-adrenergic antagonists, such as timolol), or amiloride analogs, as well as, but not limited to, the many compounds produced by Hoechst, *i.e.*, cariporide, as well as other compounds that would be recognized as modulators of Na^+ uptake or the anion exchange system. See, *e.g.*, Scholz *et al.*, *Cardiovascular Research* 29:260-268 (1995). Included within the families of drugs are analogs and new compounds, which represent improvements to the known compounds. Collectively, these compounds are referred to herein as the "modulating" drugs or compounds. Note that recent data (Figures 2-6) indicate, for the first time, that in glaucoma the clinical effects of β-blockers may arise from cyclic AMP-independent inhibition of the Na^+/proton antiport.

In the present invention, a pharmaceutical composition which upon administration increases or decreases secretion of the aqueous humor as compared to the level prior to administration, is termed a "secretion modulator;" and the amount of the modulator necessary to effect the change is termed the "secretion modulating

amount." Similarly, a pharmaceutical composition which upon administration increases or decreases fluid pressure in the aqueous humor or intraocular pressure, as compared to the level prior to administration, is termed a "pressure modulator;" and the amount of the modulator necessary to effect the change is termed the "pressure modulating amount." In accordance with the present invention, "administration" refers to administration of the modulator to cells, *e.g.*, the ciliary epithelial cells, *in vitro* or *in vivo*. Thus, use of the modulator composition, which can include drugs, compounds, pharmaceuticals or the like, can be used to treat and individual, such as a glaucoma patient, or simply to treat the affected cells, such as those of the aqueous humor or ciliary epithelium.

Moreover, the modulating drugs or compounds can be used alone or in combinations of two or more compounds. For example although ineffective alone, the simultaneous addition of both bumetanide and DIDS did inhibit the RVI.

In accordance with the present invention, videomicroscopy of BCECF-loaded PE cells verified the presence of an EIPA-inhibitable Na^+/H^+ antiport. Removing external Cl^- also triggered an alkalinization, which was Na^+-independent and DIDS-inhibitable.

Moreover, application of hypotonicity followed by return to isotonicity triggered a regulatory volume increase, which was pharmacologically similar to the uptake mechanisms described for intact rabbit ciliary epithelium. RT-PCR amplification of RNA from human ciliary body detected expression of the AE2 Cl^-/HCO_3^- exchanger, but not of AE1, cAE3 or bAE3. Immunostaining of bovine PE cells also revealed the presence of AE2 epitope.

Thus, it is clear that the paired NHE-1 Na^+/H^+ and AE2 Cl^-/HCO_3^- antiports are important components in the initial step in aqueous humor formation, and based upon this knowledge, treatments and prevention measures can be taken to manage elevated intraocular pressure through the use of drugs or compounds that modulate the effect of the antiports.

NHE-1. The measured values of the apparent Ki for EIPA, cariporide and amiloride conform to the known values for the NHE-1 Na^+/H^+ exchanger, and differ substantially from those characterizing NHE-2 and NHE-3 (Table 5 in Example 3). It will be noted that the relative apparent Ki of NHE-1 is six-fold lower than that of

NHE-2 for the amiloride analogues EIPA and MPA, and 50-fold lower for the Hoechst inhibitors. The apparent Ki values of NHE-3 are 1-2 orders of magnitude higher for each of the three sets of inhibitors (the amiloride analogues, the Hoechst compounds and amiloride, itself). Thus, the measurements of $^{22}Na^+$ uptake (Figure 4) uniquely identify the functional activity of the NHE-1 Na^+/H^+ antiport.

The data obtained with brief trypsinization indicates that NHE-1 is largely expressed in the basolateral equivalent membranes of bovine PE cells grown on glass coverslips (Figure 11). This interpretation is consistent with findings in most cells (Coupaye-Gérard *et al.*, *Am J Physiol* 271:C1639-C1645 (1996)), although NHE-1 is expressed in both the basolateral and apical membranes of some cell lines (Helmle-Kolb *et al.*, 1993).

AE2. The fluoro-videomicroscopic measurements of Figures 12-14 document the reversible, DIDS-inhibitable, Na^+-independent Cl^-/HCO_3^- exchange (Figure 12). Interestingly, in an earlier careful study, Butler *et al.*, *Exp Eye Res* 59:343-349 (1994), observed Na^+-dependent, but not Na^+-independent Cl^-/HCO_3^- exchange by native PE cells. The precise basis for this difference is unclear, but there were many differences in approach between the two studies. Butler *et al.* applied videomicroscopy to separated sheets of PE cells from the rabbit ciliary epithelium studied on a heated stage, whereas the data shown in Figures 12-14 were obtained with cultured bovine PE cells at room temperature.

The findings of the present invention regarding the inhibitable, Na^+-independent Cl^-/HCO_3^- exchangers, were verified by immunochemical analysis that bovine PE cells displayed AE2 polypeptide (Figure 15). Moreover, as illustrated in Figure 19, messenger RNA for the best characterized family of Na^+-independent Cl^-/HCO_3^- exchangers (the "AE anion exchangers") were found in preparations of human ciliary bodies. RT-PCR amplification from human ciliary body detected mRNA uniquely for AE2, and not AE1, bAE3 or cAE3. Although AE2 is commonly localized to the basolateral membranes of other polarized cell types, it may be detected rarely in both membranes (Alper *et al.*, 1999). The lack of effect of trypsin on AE activity (Figure 14) suggests either that anion exchanger polypeptide (likely AE2) is not restricted to plasma membrane adjacent to the glass substratum, or that Cl^- gains access better than Na^+ to the equivalent lateral membrane of these cells.

Potential physiologic implications. The NHE-1 isoform of the Na^+/H^+ exchangers is ubiquitously expressed in all eukaryotic cells (Counillon *et al.*, *J. Biol Chem* 275:1-4 (2000) and Cl^-/HCO_3^- exchange is present in nearly all tissues and cells (Alper, 1994). However, such exchange can subserve intracellular pH regulation, without contributing to transepithelial transport. The data shown in Figure 15 establish that cell shrinkage can trigger uptake of solute and fluid by the PE cells (the post-RVD RVI). This fluid uptake can be inhibited by blocking the Na^+/H^+ antiport with dimethylamiloride (Figure 16) or by blocking Cl^-/HCO_3^- exchange by omitting CO_2/HCO_3^- (Figure 17). When the Na^+-K^+-$2Cl^-$ symport is blocked with bumetanide, the further addition of DIDS also blocks the post-RVD RVI (Figure 17). Thus, the paired exchange of NHE-1 and AE2 can lead to net fluid uptake from the extracellular compartment into the PE cells, as demonstrated in other systems (Jiang *et al.*, *Am J Physiol* 272:C191-202 (1997)).

Accordingly, the findings of the present invention are consistent with data showing that the paired Na^+/H^+ and Cl^-/HCO_3^- antiports (Kaufman *et al.*, 1994; McLaughlin *et al.*, 1998; Wiederholt *et al.*, 1991) play an important role in the first stage of aqueous humor formation, uptake of NaCl from the stroma of the ciliary processes, as can the bumetanide-sensitive Na^+-K^+-$2Cl^-$ symport (Edelman *et al.*, 1994; To *et al.*, *Curr Eye Res* 17:896-902 (1998); Wiederholt *et al.*, 1986). The presently discovered importance of the paired operation of the NHE-1 and AE2 exchangers also explains the clinical efficacy of carbonic anhydrase inhibitors in treating glaucoma (Kaufman *et al.*, 1994; McLaughlin *et al.*, 1998; Wiederholt *et al.*, 1991). Reducing the availability of H^+ and HCO_3^- to the antiports thereby inhibits the initial step in aqueous humor secretion. The current data suggest that this step could be more selectively blocked in glaucomatous patients by specifically inhibiting NHE-1 with low concentrations of EIPA, DMA or cariporide, particularly in combination with bumetanide to simultaneously block the symport.

Modulating compounds of the present invention will be administered to the eye in amounts and over a schedule effective to raise the intraocular pressure of the eye, particularly when the intraocular pressure was previous reduced or depressed, *i.e.* below about 20 mmHg, usually below 18 mmHg, and more usually below 8 mmHg, or when the eye suffers from hypotonia for any reason. The amount of the compound

required for such pressure increase and/or hypotonia alleviation will depend on a number of factors, including the initial pressure, condition of the pertinent activity of the administered compound, and the like, with exemplary amounts typically being in the range from about 50 μg to 5 mg per dose (*i.e.*, single application of the composition) usually being from 250 μg to 1 mg per dose.

For systemic administration, the dosage of the agents according to this invention generally is between about 0.1 μg/kg and 10 mg/kg, preferable between 10 μg/kg and 1 mg/kg. For topical administration, dosages of between 0.000001% and 10% of the active ingredient are contemplated, preferably between about 0.1% and 4%. It will be appreciated that the actual preferred amounts of agent will vary according to the specific agent being used, the severity of the disorder, the particular compositions being formulated, the mode of application and the species being treated. Dosages for a given host can be determined using conventional considerations, *e.g.*, by customary comparison of the differential activities of the subject compounds and of a known agent, *e.g.*, by means of an appropriate, conventional pharmacologic protocol. The agents are administered from less than once per day (*e.g.*, every other day) to four times per day.

Such dosages may be conveniently achieved using compositions having the compound present in a suitable ophthalmically acceptable carrier at a concentration in the range from about 0.1 weight percent to 5 weight percent. Concentrations above 5 weight percent are potentially toxic and should generally be avoided. Specific formulations will be prepared in accordance with standard principles in the art, or as exemplified below.

It is also be possible to incorporate the modulating compounds of the present invention into controlled-release formulations and articles, where the total amount of compound is released over time, *e.g.*, over a number of minutes or hours. Typically, the total dosage of the compound will be within the limits described above for non-controlled-release formulations, but in some cases may be greater, particularly when the controlled release formulations act over relatively longer periods of time. Suitable controlled release articles for use with the compositions of the present invention include solid ocular inserts of the type available from commercial vendors.

Other controlled-release formulations may be based on polymeric carriers, including both water-soluble polymers and porous polymers having desirable controlled-release characteristics. Particularly suitable polymeric carriers include various cellulose derivatives, such as methylcellulose, sodium carboxymethylcellulose, hydroxyethylcellulose, and the like.

Suitable porous polymeric carriers can be formed as polymers and copolymers of acrylic acid, polyacrylic acids, ethylacrylates, methylmethacrylates, polyacrylamides, and the like. Certain natural biopolymers may also find use, such as gelatins, alginates, pectins, agars, starches, and the like. A wide variety of controlled-release carriers are known in the art and available for use with the present invention.

Topical compositions for delivering the modulating compounds of the present invention will typically comprise the compound present in a suitable ophthalmically acceptable carrier, including both organic and inorganic carriers. Exemplary ophthalmically acceptable carriers include water, buffered aqueous solutions, isotonic mixtures of water and water-immiscible solvents, such as alkanols, arylalkanols, vegetable oils, polyalkalene glycols, petroleum-based jellies, ethyl cellulose, ethyl oleate, carboxymethylcelluloses, polyvinylpyrrolidones, isopropyl myristates, and the like. Suitable buffers include sodium chloride, sodium borate, sodium acetate, gluconates, phosphates, and the like.

The formulations of the present invention may also contain ophthalmically acceptable auxiliary components, such as emulsifiers, preservatives, wetting agents, thixotropic agents (*e.g.*, polyethylene glycols, antimicrobials, chelating agents, and the like). Particularly suitable antimicrobial agents include quaternary ammonium compounds, benzalkonium chloride, phenylmercuric salts, thimerosal, methyl paraben, propyl paraben, benzyl alcohol, phenylethanol, sorbitan, monolaurate, triethanolamine oleate, polyoxyethylene sorbitan monopalmitylate, dioctyl sodium sulfosuccinate, monothioglycerol, and the like. Ethylenediamine tetracetic acid (EDTA) is a suitable chelating agent.

Modulating compounds of the present invention can be administered opthamologically, subcutaneously, intravenously, intramuscularly, topically, orally, nasally, buccally, by inhalation spray, or via an implanted reservoir. In a preferred embodiment, the therapeutic agent is administered to the eye, such as by topical

administration (e.g., eye drops or emulsion). They can be administered in dosage formulations containing conventional non-toxic pharmaceutically-acceptable carriers, adjuvants and/or vehicles.

The form in which the agents are administered (e.g., capsule, tablet, solution, emulsion) will depend at least in part on the route by which they are administered. A therapeutically effective amount of the agent is that amount necessary to significantly reduce or eliminate symptoms associated with glaucoma. The therapeutically effective amount will be determined on an individual basis and will be based, at least in part, on consideration of the agent, the individual's size and gender, the severity of symptoms to be treated, the result sought. Thus, the therapeutically effective amount can be determined by one or ordinary skill in the art, employing such factors and routine experimentation.

The therapeutically effective amount can be administered in a series of doses separated by appropriate intervals, such as hours, days or weeks. Alternatively, the therapeutically effective amount can be administered in a single dose. The term, "single dose," as used herein, can be a solitary dose, and can also be a sustained release dose, such as by a controlled-release dosage formulation of a continuous infusion. Other drugs can also be administered in conjunction with the agent.

The present invention is further described in the following examples. These examples are not to be construed as limiting the scope of the appended claims.

EXAMPLES

Example 1. The Role of the Sodium/Proton Exchanger (Antiport) in the Uptake of Salts and Fluids into the Aqueous Humor

Electrophysiological, volumetric and molecular biological studies were conducted to evaluate the activity of the chloride channels on the aqueous surface of the NPE cells to determine their role in the rate of formation of the aqueous humor. This is because once the channels can be selectively controlled, the rate of release of chloride and water can be controlled from the NPE cells to the aqueous humor. Elevated intraocular pressure, such as that which typifies glaucoma could then be treated by the transfer of anions, such as cyclamate, into the aqueous humor to block the native chloride channels.

The first step in the formation of the secretion of the aqueous humor was examined by electron-probe X-ray microanalysis, a biophysical method for quantifying the intracellular content of chloride, sodium and potassium in the isolated rabbit ciliary epithelium in accordance with the methods of Bowler *et al.*, *Exp Eye Res* 62:131-139 (1996) and McLaughlin *et al.*, 1998. This method is particularly advantageous because it is uniquely capable of quantifying the Na, K and Cl contents at the visualized sites within individual cells (*e.g.*, Civan, (1983), Chapter 6, In: Epithelial Ions and Transport: Application of Biophysical Techniques, Wiley, New York.

Cellular Model Dutch-belted rabbits of either sex and older than 6 weeks post-weaning were obtained from the Department of Laboratory Animal Sciences, University of Otago Medical School, and were treated in accordance with the ARVO Resolution on the Use of Animals in Research. The animals were anaesthetised with 30 mg/kg sodium pentobarbital and sacrificed by injecting air into the marginal ear vein. After enucleation, the iris-ciliary body was excised, cut into quarters and each quarter bonded at its edge to plastic frames with cyanoacrylate. Dissected tissue was then incubated for at least 2 hours in either bicarbonate or bicarbonate-free medium. Pairs of quadrants (one from each eye) were then incubated separately at room temperature (18-22°C) in a beaker for at least 30 minutes under the different experimental conditions. Incubations were conducted at room temperature (18-22°C) for the reasons discussed in McLaughlin *et al.*, 1998.

After incubation, the tissues were blotted and a 30% albumin solution was applied briefly to the epithelial surface of the NPE cells (*i.e.*, to the basement membrane supporting the NPE cells). Excess albumin was removed by blotting and the tissue segment was then plunged into liquid propane at -180°C to freeze the preparation quickly before ions and water could undergo redistribution. Sections were cut to 0.2-0.4 μm in thickness at -80 to -90°C with a cryoultramicrotome, freeze-dried at 10^{-4} Pa (equivalent to 7.5×10^{-7} Torr), and transferred for analysis to a scanning electron microscope (JEOL JSM 840) equipped with an energy-dispersive spectrometer.

Unless otherwise stated, between 5 and 8 pairs of NPE and PE cells were measured in each of two sections cut from each quadrant.

Solutions and chemicals. The bicarbonate medium contained (in mM): 145 Na^+, 5.9 K^+, 122.1 Cl^-, 15.0 HEPES [4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid], 1.2 Mg^{2+}, 2.5 Ca^{2+}, 1.2 $H_2PO_4^-$, 30 HCO_3^- and 10 glucose at pH 7.30-7.45 and 305-315 mosmol. Bicarbonate-free solution was prepared by isosmolar replacement of HCO_3^- by Cl^-. Depending on whether or not HCO_3^- was included, the gas bubbled through the solution throughout incubation consisted of either 95%O_2-5%CO_2 or pure O_2, respectively. All chemicals were reagent grade. Bovine albumin (RIA grade, Immuno Chemical Products) was dialysed for 48-60 hours, freeze-dried at -70°C, and stored at 4°C. A 30% (w/v) solution was prepared by dissolving the albumin in the same medium in which the tissue was incubated. Timolol and dimethylamiloride were added to the incubation media from stock solutions in water. Dibutyryl cyclic adenosinemonophosphate was dissolved directly in incubation media. Acetazoleamide was added from stock solutions in dimethylformamide, with the same concentration of solvent vehicle (0.1% vol/vol) applied to the parallel control preparations.

Data acquisition and reduction. Electron probe X-ray microanalysis was selected because it permits both quantification and localization of intracellular elements. Using an electron microscope, a specific visualized area was targeted within the cell. The specimen was irradiated with a beam of electrons, which ionizes a small fraction of the atoms bombarded. In principle, after an electron is knocked from an inner atomic shell, an electron from an outer shell can take its place. The relaxation of the electron from a higher to a lower energy state generates a quantum of X-ray energy. Spectroscopic measurement of the characteristic energy and number of these quanta permits identification and quantification of the elements within the sample.

The dried sections were imaged with a transmitted electron detector. Measurements were collected with a Tracor Northern X-ray 30 mm^2 detector, using a probe current of 140-200 pA for 100 seconds at an accelerating voltage of 20 kV. The intracellular data were obtained by the electron beam scanning a rectangular area within the nucleus of each selected NPE or PE cell which varied from ~0.9 x 1.2 μm to ~2.4 x 3.0 μm depending on the size of the nucleus analyzed.

The elemental peaks were quantified by filtered least-square fitting to a library of mono-elemental peaks (Bowler *et al.*, *J Membrane Biol* 123:115-132 (1991)). The library spectra for Na, Mg, Si, P, S, Cl, K and Ca were derived from microcrystals sprayed onto a Formvar film. White counts were summed over the range 4.6-6.0 keV, and corrected for the non-tissue contributions arising from the Al specimen holder and Ni grid.

For purposes of data reduction the elemental peaks were routinely normalized to the phosphorus signal obtained in the same scanned area of each cell. The reported values reported for Na/P, Cl/P and K/P were the measured estimates of the intracellular Na, Cl and K contents, respectively. Although it is not possible to calculate ion concentrations in mmol/L from these data, the intracellular contents of (Na + K) or of (Na + K + Cl) provide indices of intracellular water content (Abraham *et al.*, *Am J Physiol* 248:C154-C164 (1985)). For this reason, the measured values of (Na + K)/P and of (Na + K + Cl)/P are also entered in the Tables.

In Figures 2-6, values are presented as the means ±1 SE. The number of cells analyzed are indicated by the symbol "n." Figures 2-6 are presented in box plots, permitting the presentation of all data points (see, *e.g.*, the description of Figure 2. In these experiments the differences between more than two groups of data have been analyzed by ANOVA, using non-parametric (Kruskal-Wallis) testing. The probabilities of the null hypothesis have been calculated with the Dunn Multiple Comparisons post-test. With two groups, the non-parametric Mann Whitney test was used.

Effects of timolol on epithelial cell composition in tissues incubated in the presence or absence of HCO_3^-/CO_2 solution. Timolol was applied at a concentration of 10μM, within the range of concentrations likely reached clinically in the aqueous humor. Conjunctival instillation of 20-50μl of 0.5% timolol into the rabbit conjunctival sac can be calculated to produce peak concentrations of ~8μM (Vareilles *et al.*, *Ophthalmol Vis Sci* 16:987-996 (1977)) to 17μM (Ohtori *et al.*, *Exp Eye Res* 66:487-494 (1998)). The same concentration has been used in other *in vitro* studies of timolol's mode of action (Krupin *et al.*, 1991). In the absence of HCO_3^-/CO_2 timolol produced no significant changes in epithelial cell Na, Cl or K.

In contrast, in ciliary tissue from the same eyes incubated in HCO_3^-/CO_2, 10 μM-timolol resulted in significant losses of Cl and K (Figure 2). A time course was obtained in a separate experiment conducted with HCO_3^-/CO_2 solution (Figure 3). Significant losses of Cl ($P< 0.001$ and K ($P< 0.05$) were detected by 10 minutes (Figure 3). Both Cl and K remained below control levels over the subsequent 30 minutes, but the K loss was not statistically significant at 20 and 40 minutes.

For each condition 8 sections were analysed, with 6 NPE and 6 PE cells measured in each section, giving a total of 96 cell measurements for each condition. Altogether the data was obtained from 32 eyes in which tissues were incubated in bicarbonate with or without timolol for 20–30 minutes (Table 1). There were highly significant ($P< 0.001$), comparable losses of Cl/P (-0.059 ± 0.006) and K/P (-0.064 ± 0.010), in the presence of bicarbonate (bicarb., also HCO_3^-)/ CO_2 (Figure 4).

Table 1. Effects of timolol in HCO_3^-/CO_2 solutions, all available results. (Data from 80 sections from 16 animals.)

Table 1. Effect of timolol on ciliary composition

Composition	n	Na/P	Cl/P	K/P	Na+K/P	Na+K-Cl/P	Na+K+Cl-P
Control	346	0.097 ± 0.003	0.312 ± 0.005	1.143 ± 0.008	1.240 ± 0.009	0.928 ± 0.006	1.552 ± 0.013
+ timolol	570	0.092 ± 0.002	0.253 ± 0.003	1.079 ± 0.006	1.171 ± 0.006	0.918 ± 0.005	1.424 ± 0.008
Difference		- 0.005 ±0.004	- 0.059 ±0.006	- 0.064 ±0.010	-0.069 ±0.011	-0.010 ±0.009	-0.128 ±0.015
P		NS	<0.001	<0.001	<0.001	NS	<0.001

In contrast, timolol was without effect in the absence of external carbon dioxide and bicarbonate (Table 2).

Table 2 (Parts A and B). Effect of timolol on ciliary composition, in the presence or absence of external bicarbonate.

Table 2A. Effect in bicarbonate-free solution.

Composition	n	Na/P	Cl/P	K/P
Control	96	0.086 ± 0.005	0.233 ± 0.006	1.0 ± 0.011
+ timolol	96	0.090 ± 0.002	0.215 ± 0.002	1.002 ± 0.004
Differences		+ 0.004	- 0.018	+ 0.002

Table 2B. Effect of bicarbonate in solution.

Composition	n	Na/P	Cl/P	K/P
Control	96	0.099 ± 0.006	0.312 ± 0.008	1.115 ± 0.015
+ timolol	96	0.112 ± 0.005	0.269 ± 0.007	1.063 ± 0.015
Differences		+ 0.013	- 0.043	+ 0.052

Effects of cAMP on epithelial cell composition in tissues incubated in HCO_3^-/CO_2 solution. As a known β-blocker, current knowledge indicates that timolol would be expected to act by reducing cell cAMP levels, and the blocking action should be circumvented by directly adding a membrane-permeant form (dibutyryl cAMP) of cAMP. Accordingly, to verify the presumed mechanism, the effects of a cAMP analogue were analyzed on an epithelial cell composition, alone or with timolol (Figure 4).

Data were obtained from experiments using eyes from two animals: for controls, 8 sections were analysed, with 6-7 NPE and PE cells measured in each section, giving a total of 98 cell measurements; for timolol, 8 sections were analysed, with 6 NPE and PE cells measured in each section, giving a total of 96 cell measurements; for cAMP, 8 sections were analysed, with 6 NPE and PE cells measured in each section, giving a total of 96 cell measurements; for timolol +cAMP, 10 sections were analysed, with 3-6 NPE and PE cells measured in each section, giving a total of 96 cell measurements.

Dibutyryl cyclic AMP (1mM) was without effect on cell Cl but reduced cell K significantly. Timolol (10μM) reduced both Cl and K significantly. The combination of the two agents appeared to be additive, with Cl lost as expected for timolol alone, and a loss of K that was twice as great as the loss of Cl.

As shown in Table 3, in contrast to the actions of timolol, cAMP was found to have no significant effects on the chloride content in the presence of bicarbonate. In fact, timolol produced quantitatively the same reduction in chloride content, whether of not cAMP was present, unequivocally demonstrating that timolol is not acting through the cAMP system - contrary to conventional wisdom.

Table 3. Effects of timolol and cAMP in a bicarbonate-containing solution.

no.	Na/P	Cl/P	K/P	Na+K/P	Na+K-Cl/P	Na+K+Cl/P
NaCl-R(+ bicarb) (control)						
98	0.093 0.006	0.307 0.009	1.145 0.016	1.237 0.017	0.930 0.012	1.544 0.025
NaCl-R(+bicarb) +timolol						
96	0.104 0.006	0.270 0.006	1.058 0.013	1.162 0.015	0.892 0.012	1.432 0.020
timolol – control	-0.012	-0.037	-0.087	0.075	-0.039	-0.112
NaCl-R(+bicarb) +cAMP						
96	0.094 0.006	0.306 0.008	1.092 0.013	1.186 0.014	0.880 0.011	1.492 0.020
cAMP – control	0.002	-0.001	-0.053	0.052	-0.050	-0.053
NaCl-R(+bicarb) +cAMP + timolol						
96	0.113 0.006	0.251 0.006	1.029 0.012	1.142 0.013	0.891 0.009	1.393 0.018
both – control	0.020	-0.056	-0.115	0.095	-0.039	-0.152

Effects of acetazolamide and timolol on epithelial cell composition in tissues incubated in HCO_3^-/CO_2 solution. Previous studies have shown that the carbonic anhydrase inhibitor, acetazolamide, decreases cell Cl and K (Bowler *et al.*, 1996; McLaughlin *et al.*, 1998). Therefore, since timolol also decreased cell Cl and K, the effects of acetazolamide and timolol were compared (Figure 5).

Data were obtained from experiments using eyes from two animals: for controls, 5 sections were analysed, with 3-6 NPE and PE cells measured in each section, giving a total of 54 cell measurements; for timolol, 7 sections were analysed, with 6-7 NPE and PE cells measured in each section, giving a total of 86 cell measurements; for acetazolamide, 4 sections were analysed, with 6 NPE and PE cells measured in each section, giving a total of 48 cell measurements. For timolol +acetazolamide, 6 sections were analysed, with 6-7 NPE and PE cells measured in each section, giving a total of 74 cell measurements.

As shown in Figure 5, Cl/P was decreased by 0.5 mM-acetazolamide (-0.123 ±0.012) to a greater extent than it was by 10 μM-timolol (-0.045 ±0.013). However, the two effects were not additive, for the combination of inhibitors caused no greater statistically significant reduction of Cl/P (-0.104 ±0.021) than did acetazolamide alone.

Effects of dimethylamiloride on epithelial cell composition in tissues incubated in HCO_3^-/CO_2 solution. Since it was possible that timolol was affecting some aspect of H^+/Na^+ and HCO_3^-/Cl^- exchange, the effects of a known inhibitor of H^+/Na^- exchange, dimethylamiloride (Figure 6) were examined. Data were obtained from experiments using eyes from two animals: for each condition 8 sections were analysed, with 6-7 NPE and PE cells measured in each section, giving a total of 98 cell measurements for each condition.

In separate measurements, the effect dimethylamiloride (50 μM) was similar to that of 10 μM-timolol, with significant reductions in cell Cl and K. See Table 4.

Table 4. Effect produced by a known inhibitor (dimethylamiloride) of the Na/H^+ exchanger.

no.	Na/P	Cl/P	K/P	Na+K/P	Na+K-Cl/P	Na+K+Cl/P
Na bicarb-R control						
96	0.110 0.007	0.341 0.008	1.130 0.015	1.240 0.017	0.899 0.013	1.581 0.023
Na bicarb-R+DMA 20 min						
100	0.095 0.007	0.283 0.007	1.069 0.014	1.163 0.015	0.880 0.011	1.446 0.020
DMA – control	-0.015	-0.058	-0.062	0.077	-0.019	-0.135

Example 2. Use of Anions to Block the Chloride Channels

On the basis of electrophysiological, volumetric, and molecular biological observations, a model has been formulated in which the activity of the chloride channels on the aqueous surface of the nonpigmented ciliary epithelial (NPE) cells is considered to be a major factor limiting the rate of formation of the aqueous humor. If these channels are selectively inhibited, the rate of release of chloride, and secondarily water, from the NPE cells into the aqueous humor can be limited.

One of the major means of activating these chloride channels is through the normal metabolite adenosine (Carré *et al.*, 1997). For example, in the attached Figure 8, the current carried by chloride ion in the presence of an activator (IB-MECA) of the adenosine receptor has been measured as a function of the voltage applied across the cell membranes of immortalized cultured NPE cells. This calculation was performed by measuring the currents at a given voltage after stimulating the receptor and subtracting off the baseline values at the same voltages, using the ruptured-patch

whole-cell patch-clamp technique. In the presence of chloride, the adenosine activator produced a large increase in current , as compared with the currents shown in Figure 7.

Note that in Figures 7-9, the null hypothesis, that the experimental and baseline measurements shared the same mean and distribution, was tested with Student's t-test and by the upper significance limits of the F-distribution, as indicated. The t-test was applied to compare the significance between single means or single fit parameters. The F-distribution was applied to test whether the time course of volume measurements in different suspensions could reflect a single population of data points.

On the other hand, when part of the external chloride was replaced by the normal anion component of human aspartate, the positive current (upwards) going out of the cell was reduced, while the inward current is unchanged (Figure 7). This was expected since the aspartate is larger than chloride and is expected to move more slowly from outside the cell into the cell. The inward movement of a negative ion (like chloride) is responsible for the outward positive current. Since the composition of the inner solution did not change, changes were not expected in the outward movement of chloride at a given voltage, measured as the inward positive current.

The situation was quite different for the IB-MECA stimulated chloride current in the presence of cyclamate. The concentration of chloride was 25 mM inside and outside the cell in Figure 7. Both curves were also obtained with 91 mM of the additional anion outside. However, in contrast to aspartate, cyclamate at the same concentration reduced both the outward and the inward currents substantially. Thus, the reduction in inward current reflects a previously unknown block of the chloride currents of NPE cells by anions. As a result, simple cyclamates, of the type used in foods, but not limited to only such cyclamates, can actually block the chloride channels need to form the aqueous humor, thereby aiding in the control and regulation of elevated intraocular pressure.

Example 3: The Control of Sodium/Proton Exchangers to Control the Secretion of Excess Fluids into the Aqueous Humor.

Cellular model. The cells studied were an immortalized PE-cell line developed by the inventors from a primary culture of bovine pigmented ciliary

epithelium, previously characterized by several research groups (*e.g.*, Mitchell *et al.*, *Invest Ophthalmol Vis Sci* 38 (Suppl.):S1042 (1997); Wax *et al.*, *Exp Eye Res* 57:89-95 (1993)). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, #11965-027, Gibco BRL, Grand Island, NY; and 51-43150, JRH Biosciences, Lenexa, KS) with 10% fetal bovine serum (FBS, A-1115-L, HyClone Laboratories, Inc., Logan, UT) and 50 μg/ml gentamycin (#15750-011, Gibco BRL), at 37°C in 5% CO_2 (Yantorno *et al.*, *Exp Eye Res* 49:423-437 (1989)). The medium had an osmolality of 328 mOsm. Cells were passaged every 6-7 days and, after reaching confluence, were suspended in solution for study within 6-10 days after passage.

Pharmacological characterization of the Na^+/H^+ exchanger. By the method of Counillon *et al.*, *Mol Pharmacol* 44:1041-1045 (1993), and herein incorporated by reference, cells were seeded on 24-well plates. After overnight incubation, the cells were preincubated in the NH_3/NH_4^+ loading medium for 60 minutes. Then, they were rinsed rapidly with choline chloride-buffer, after which the $^{22}Na^+$-uptake solution was applied and the initial rates of $^{22}Na^+$-uptake were determined. After 5 minutes, influx was stopped by rapidly rinsing the cells (four times within <10 seconds) with ice-cold phosphate-buffered saline (PBS: 150mM NaCl and 5mM sodium phosphate at pH 7.4). Thereafter, the cells were solubilized in 0.1M NaOH, and the $^{22}Na^+$ level was determined with a γ-counter.

The data were taken from 3 experiments, with each assay conducted in duplicate and each reading obtained with two windows. The apparent Ki value for each of the inhibitors was estimated from a linear least-squares analysis of V_{max}/v as a function of inhibitor concentration (c), where V_{max} was the maximal rate of uptake and v was the uptake at any concentration c.

Fluorescence experiments. Intracellular pH (pH_c) was measured using the pH-sensitive fluorescent dye BCECF (Bidet *et al.*, *Pflügers Arch* 416:270-80 (1990); Helmle-Kolb *et al.*, *Pflügers Arch* 425:34-40 (1993)). Cells grown in Petri dishes were loaded for ~10 min with 1μM of the pentaacetoxy-methyl ester of BCECF, at 37°C. Na^+/H^+ exchange and Cl^-/HCO_3^- exchange were separately monitored under constant superfusion by the optical system described below.

Na^+/H^+ exchange activity was examined after imposing an acid load with an NH_3/NH_4^+ prepulse in the absence of added bicarbonate (Boron *et al.*, *J Gen Physiol*

67:91-112 (1976)). Cl^-/HCO_3^- exchange activity was monitored by maintaining the HCO_3^- concentration of the superfusate constant (at 5-10 mM) during the course of intermittently removing external Cl^-.

Image analysis. The approach of Rubera *et al.*, *Am J Physiol* 273:F680-F697 (1997)) was followed for analyzing the images. The optical system was composed of a Zeiss ICM405 inverted microscope and a Zeiss 40 objective that was used for epifluorescent measurements with a 75 W xenon lamp. The excitation beam was filtered through narrow-band filters (490 and 450 nm (Oriel, Conn, USA)), mounted in a motorized wheel (Lambda 10-2, Sutter Instrument Co., CA, USA) equipped with a shutter in order to control the exposure times.

The incident and the emitted fluorescence radiation beams were separated through a Zeiss chromatic beam splitter. Fluorescence emission was selected through a 530 nm narrow-band filter (Oriel, Conn., USA). The transmitted light images were viewed by an intensified camera (Extended ISIS , Photonic Science Ltd., Sussex, UK). The 8 bit extended ISIS camera was equipped with an integration module in order to maximize signal to noise ratio. The video signal from the camera proceeded to an image processor integrated in a DT2867 image card (Data Translation®, MA, USA) installed in a Pentium 100 PC computer. The processor converts the video signal into 512 lines by 768 square pixels per line by 8 bits per pixel. The 8-bit information for each pixel represents one of the 256 possible grey levels ranging from 0 (for black) to 255 (for white). Image acquisition and analysis were performed by the 2.1 version of AIW software (Axon Instr., CA, USA). The final calculations were made with a software program developed within the inventor's laboratory using the Excel software (Microsoft Corp., WA, USA) (Touret, (1997) Etude des relations structure-fonction de l'échangeur Na+/H+. Diplôme d'Études Approfondies de Biologie Cellulaire et Moleculaire, Université de Nice-Sophia antipolis).

Volumetric measurements and analysis. After harvesting cells from a T-75 flask by trypsinization (Yantorno *et al.*, 1989), a 0.5-ml aliquot of the cell suspension in DMEM was added to 20 ml of each test solution (Table 5).

Table 5. Pharmacologic profiles of NHE antiports

Inhibitor	PE cells	NHE-1	NHE-2	NHE-3
Amiloride Analogues				
EIPA	0.068 ±0.002	0.05**		
Methylpropylamiloride*		0.08	0.5	10
Hoechst Compounds.				
Cariporide	0.25 ±0.02	0.1**		
HOE694**		0.2	10	650
Amiloride*	3.9 ±0.2	3	1-3	100

*Counillon *et al.*, 1993.

**Scholz *et al.*, *Cardiovascular Research* 29:260-268 (1995).

Parallel aliquots of cells were studied on the same day. One aliquot usually served as a control, and the others were exposed to different experimental conditions at the time of suspension. The same amount of solvent vehicle (dimethylformamide, DMSO or ethanol) was always added to the control and experimental aliquots. The sequence of studying the suspensions was varied to preclude systematic time-dependent artifacts (Civan *et al.*, *Invest Ophthalmol Vis Sci* 35:2876-2886 (1994)). Cell volumes of isosmotic suspensions were measured with a Coulter Counter (model ZBI-Channelyzer II), using a 100-μm aperture (Civan *et al.*, *Exp Eye Res* 54:181-191 (1992)). As previously described (Yantorno *et al.*, 1989), the cell volume (v_c) of the suspension was taken as the peak of the distribution function.

Reverse transcriptase-phosphorylase chain reaction (RT-PCR). Total RNA was extracted from a human ciliary body by the guanidine HCl method (Escribano *et al.*, *J Biochem* (Tokyo) 118(5):921-931 (1995)). The ocular tissue was obtained through the National Disease Research Interchange (Philadelphia, PA) from a 65 year old cadaver eye donor, with no past history of eye disease, within 24 h after enucleation. RNA (0.25μg) was reverse transcribed using the RETROscript kit (Ambion, Woodlands, TX). cDNA was subjected to hot start PCR. PCR mixes lacking only primers were preheated at 82°C for 1 minute. Then, gene-specific primers (Loffing *et al.*, submitted, 1999) primer sequences available upon request) were injected into the mix through oil.

The complete reaction mixes were denatured for 3 minutes at 95°C, then cycled through the following conditions: 45 seconds of denaturation at 94°C, 2 minutes of annealing at 60°C, and 2min of elongation at 72°C. After 42 cycles, final extension of 10 minutes at 72°C was terminated by rapid cooling to 4°C. PCR products were separated in 1% agarose gels and visualized by ethidium bromide staining.

The amount of starting RNA was less than usual to avoid the mellanin-associated inhibition of *in vitro* cDNA formation obtained with RNA extracted from the ciliary body (Ortego *et al.*, *J Neurochem* 69(5):1829-1839 (1997)). The high PCR cycle number was chosen both to compensate for reduced input RNA, and to maximize sensitivity of detection.

Immunocytochemistry. Bovine PE cells were grown to confluence on glass coverslips, fixed in a 3% paraformaldehyde solution containing 140 mM NaCl and 20 mM Na phosphate at pH 7.4 (PBS) for 30 minutes, then rinsed and quenched in PBS containing 50 mM lysine at pH 8.0 for three 5-minute periods. Coverslips were immunostained with SDS epitope unmasking as described by Alper *et al.*, Am J Physiol 273:F601F614 (1997)), using affinity-purified polyclonal rabbit antibody to the C-terminal amino acids 1224-1237 of mouse AE2, in the presence of 24 μg/ml peptide antigen or C-terminal peptides of other anion exchange (AE) gene products (Alper *et al.*, *Am J Physiol* 277:G321-G332 (1999); Alper *et al.*, 1997).

Additional experiments were performed with affinity-purified antibodies to C-terminal peptides from mouse AE1 amino acids 917-929 and from human bAE3 (1216-1227). Secondary antibody was Cy3-coupled goat anti-rabbit Ig (Jackson Immunochemicals). Slides were examined by laser confocal fluorescence microscopy with a BioRad MRC 1024 confocal microscope.

Drugs and experimental solutions. Amiloride, DIDS, nigericin, EIPA, amiloride, ouabain and dimethyl sulfoxide were obtained from Sigma. BCECF/AM (2,7-biscarboxyethyl-5(6)-carboxyfluorescein pentaacetoxymethyl ester) was obtained from Molecular Probes, Inc. (Eugene, Oregon, USA). Trypsin was obtained from Gibco BRL, $^{22}Na^{+}$ from Amersham, and cariporide (HOE642) was provided by Dr. W. Scholz.

The calibrating solution for BCECF consisted of: 140 mM KCl, 1 mM $CaCl_2$, 20 mM HEPES, and 10 μM nigericin at pH values of 6.5, 7.0, 7.5, and 8.0.

The solutions used for the fluorovideomicroscopic studies of Cl^-/HCO_3^- exchange, listed in Table 6, displayed osmolalities of 293-320mOsm. In the fluoro-video-microscopic study of Na^+/H^+ exchange, HCO_3^- was omitted from the NaCl solution.

Table 6. Compositions of solutions for videomicroscopy of Cl^-/HCO_3^- exchange.

Component	NaCl	Na gluconate	NMDG Cl	NMDG gutamate
NaCl	130			
Na gluconate		130		
NMDG (base)			130	130
$NaHCO_3$	5-10	5-10		
Choline HCO_3			10	10
HEPES	20	20	20	20
KCl	5		5	
K gluconate		5		5
$CaCl_2$	1		1	
Ca hemigluconate		6		6
Glucose	5	5	7	7
PH	7.4	7.4	7.4	7.4

In studying $^{22}Na^+$ uptake, the NH_3/NH_4^+ loading solution contained: 50 mM NH_4Cl, 70 mM choline Cl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, 15 mM MOPS/Tris at pH 7.4. The acidifying rinse solution was the same, except for the replacement of 50 mM choline Cl for the NH_4Cl, and the use of 15 mM MOPS to buffer the pH to 7.0. The $^{22}Na^+$-uptake solution was identical to the acidifying rinse solution, except for the use of HEPES Tris to buffer at pH 7.4, the presence of 1μCi/ml $^{22}Na^+$- (~0.5mM), and the omission of KCl and addition of 1 mM ouabain to block the Na^+K^+-exchange pump.

$^{22}Na^+$ uptake. Figure 9 presents the data obtained with three inhibitors of the Na^+/H^+ antiport: EIPA (Frelin *et al.*, *FEBS* 154:241-245 (1986)), cariporide (Scholz *et al.*, 1995), and amiloride (Counillon *et al.*, 1993). The apparent Ki values generated by the fits were: 0.068 ±0.002 μM for EIPA; 0.25 ±0.02 μM for cariporide; and 3.9

±0.2 μM for amiloride. These values were then entered into Equation. 1 to construct the line fits shown in Figure 9:

$$v = V_{max} \cdot \{1 - [c / c + K_i]\} \quad (1)$$

The values obtained for K_i are uniquely characteristic of NHE-1 among the family of known isoforms of the Na^+/H^+ antiport (Counillon *et al.*, 1993: Scholz *et al.*, 1995).

Videomicroscopy of Na^+/H^+ exchange. Based upon nine acid-preloaded PE cells, Figure 10A presents a representative trace of the intracellular pH (pHi) response of cells to Na^+ restoration. An initial application of NH_3/NH_4^+ caused the cells to be alkalinized. Then, subsequent superfusion using choline Cl solution for a time (t) elicited rapid acidification of the cells. The effect when t=3 minutes is shown in Figure 10A, and when t=2 minutes is shown in Figure 10B.

After the pH_i reached a minimal value, the choline Cl solution was replaced with NaCl Ringer's solution. As anticipated, the Na^+ triggered a return of intracellular pH towards more alkaline values. However, the Na^+-dependent alkalinization appeared after a significant delay of 2-10 minutes (mean ±SE = 5.2 ±1.6min, N=5). For example, as shown in Figure 10A, the mean alkalinizing recovery appeared after a delay of ~4 min after the Na^+ was added.

Figure 10B presents the mean intracellular data obtained by averaging the results of 12 cells in a representative experiment, and demonstrating that the Na^+-dependent alkalinization (*i.e.*, the Na^+-triggered pHi recovery) was totally inhibited by 3μM EIPA. This observation verified that the pH shift of Figure 10A corresponded to the acid-triggered $^{22}Na^+$ uptake through the Na^+/H^+ antiport as disclosed.

At the conclusion of the experiment, adding NH_3/NH_4^+ was found to realkalinize the cells. The standard errors were as shown.

The consistent delay between adding Na^+ to the acid-loaded cells and activation of the Na^+/H^+ antiport was surprising, given the rapid response generally observed in other cell types. Therefore, to test whether the lag time reflected limited accessibility to the basolateral surface of the cells on glass coverslips, the experiment depicted in Figure 11 was conducted, based on the common practice of using trypsin to reduce the adhesiveness of cultured cells to the underlying surfaces of culture

dishes. Indeed, after a few minutes of transient trypsinization, the cells appeared to round up and partially separate from the dish.

In the absence of enzyme (Figure 11A), there was a lag time of ~8 minutes between acidification of 14 control PE cells that were not exposed to trypsin, and the onset of the alkalinizing response. As shown, in data obtained using 19 PE cells, brief trypsinization for 11 minutes reduced the delay to ~1 min (Figure 11B), which was less time than had been seen in any of the 5 other experiments. This striking effect of brief trypsinization is more easily appreciated in Figure 11C, wherein the data of panels A and B are presented on the same time scale, to comparatively show the faster response produced by reducing the area of attachment of the cells to the culture dish.

Videomicroscopy of Cl^-/HCO_3^- exchange. In contrast to the experiments shown in Figures 9-11, HCO_3^- was included in the experiments of the remaining Figures. Under these circumstances, based upon data produced in 10 PE cells, replacement of external Cl^- by gluconate consistently triggered a prompt alkalinization. This effect was reversed by restoring Cl^-, in some cases undershooting the pHi. This Cl^--dependent shift in pHi (*i.e.*, reversible and reproducible alkalinization) was observed even when external Na^+ was replaced by NMDG (an absence of external Na^+). The substitution of glutamate for Cl^- still produced mean alkalinization, whereas return of Cl^- to the cells triggered a return to pHi.

The alkalinization triggered by Cl^- removal was, however, partially blocked by adding 100 μM before eliciting the alkaline shift (Figure 13). Perfusion of four cells with 100 μM DIDS produced a small acidification and a subsequent blunting of the response to external Cl^- removal. After washout of DIDS, Cl^- replacement by gluconate triggered an alkaline shift of >1pH unit, a three-fold greater response than in the presence of DIDS (0.3 pH units).

By comparison, unlike the effect on Na^+/H^+ exchange, prior brief trypsinization (Figure 14A) did not alter the lag time between activation (by removing external Cl^-) and the onset of alkalinization noted without prior enzymatic treatment (Figure 14B). Figure 14A presents the mean results obtained from five cells after trypsinization for 5 minutes; while Figure 14B displays the averaged data from six cells of another dish studied on the same day, but without exposure to trypsin. The

enzyme had no evident effect on the lag time. The response to a second removal of Cl^- was blunted by including 100μM DIDS in the perfusate.

Volumetric measurements. The measurements of $^{22}Na^+$ uptake and fluorovideo-microscopy indicated that the bovine PE cells possess an NHE1 Na^+/H^+ antiport and a Na^+-independent Cl^-/HCO_3^- exchanger which can modify intracellular pH. Volumetric measurements were also performed to confirm that these antiports could function in parallel to transfer solution from the extracellular space into the cells.

Table 7. Compositions of solutions for electronic cell sorting

Component	Hypotonic	Isotonic
NaCl	30.5	110
$NaHCO_3$	30	30
HEPES	15	15
KCl	4.7	4.7
KH_2PO_4	1.2	1.2
$CaCl_2$	2.5	2.5
$MgCl_2$	1.2	1.2
Glucose	10	10
Osmolality (mOsm)	150-160	290-300

Figure 15 presents the baseline volume regulatory responses of the PE cells in suspension.. Under isosmotic conditions (290-300 mOsm, Table 7), cell volume was seen to decrease slowly and steadily over 50 minutes of observation at 34°C. Hypotonic swelling (150-160 mOsm, Table 7) initiated a regulatory volume decrease (RVD). Isontonicity was restored by adding solute at t=25 minutes, causing the cell to shrink and triggering a regulatory volume decrease (post-RVD RVI), which was significantly different from that of the isotonic control ($P<0.01$, F-distribution).

By comparison, hypertonicity alone (432-438 mOsm, prepared by adding NaCl to the isotonic solution of Table 7) produced shrinkage without triggering a regulatory response. The cells displayed a regulatory volume decrease (RVD), and the release of solute and water triggered by anisosmotic swelling. Hypertonicity shrank the cells but did not trigger a primary regulatory increase (RVI). Nevertheless,

as noted in many other cells (Hoffmann, *Curr Top Membr Transp* 30:125-180 (1987)), a secondary RVI could be elicited by first applying hypotonic shock, and then restoring isotonicity (at t=24 min, Figure 16) (post-RVD RVI).

In Figure 15, and in the succeeding Figures 15-18, insets display the RVI under control and experimental conditions at higher sensitivity and with the initial points aligned initially at the same relative volume (at t=28min).

Since paired Na^+/H^+ and Cl^-/HCO_3^- exchangers are known to contribute to the RVI in many other cells (Hoffman, 1987), the regulatory volume increase in the PE cells was examined. However, contrary to the reported results for fresh bovine cells (Walker *et al.*, 1999), the secondary RVI was not observed in the PE cells at room temperature. Consequently, the volumetric experiments were conducted at 34-37°C (results depicted in Figures 15-18). The precise time course of the baseline RVI was variable, so that the data of some experiments were better fit to an exponential (filled circles, Figure 16) and others to a linear expression (filled circles, Figure 18C). From a linear least-squares analysis, the mean ±SE rate of swelling was $17.5 \pm 2.7 \times 10^{-2}$ %/min during the baseline post-RVD RVI, as displayed in Figures 15-18.

Secondary RVI was inhibited either by blocking the Na^+/H^+ antiport with 10 μM dimethylamiloride (Figure 16, unfilled circles) or by omitting CO_2/HCO_3^- from the external solution (Figure 17, unfilled symbols). These findings are consistent with the data presented in Figures 2-3, wherein blockage of the Na^+/H^+ antiport with 10 μM dimethylamiloride was also inhibited the RVI (N=6, $P < 0.01$).

Inhibitors were added at the same time that isotonicity was restored (t=24min). Separate addition of either 10 μM bumetanide [to block the Na^+-K^+-2Cl^- symport (Haas *et al.*, *Am J Physiol* 245:C235-240 (1983). (Figure 17, filled triangles; Figure 18A, open triangles, N=9), or 500μM DIDS [to block the Cl^-/HCO_3^- exchanger (Grinstein *et al.*, *J Gen Physiol* 73(4):493-514 (1979))] (Figure 18B, open squares, N=3) did not inhibit the RVI in these experiments.

However, blocking both uptake mechanisms simultaneously by addition of both bumetanide and DIDS did inhibit the RVI (Figure 18C, open rhomboids, N=8, $P<0.05$). In addition, applying bumetanide alone in the nominal absence of CO_2/HCO_3^- was seen to produce the greatest inhibition of the regulatory volume

increase (open triangles, Figure 17 and 18A). Baseline recovery was slowed ($P<0.05$) and bumetanide then substantially inhibited the RVI ($P<0.01$).

RT-PCR amplification of AE gene products. In contrast to the members of the NHE family of antiports (Counillon *et al.*, 1993; Scholz *et al.*, 1995), the Na^+-independent Cl^-/HCO_3^- exchangers do not display especially distinctive pharmacologic profiles (Alper, *Cell Physiol Biochem* 4:265-281 (1994)). In the absence of complete information concerning the structure of the bovine anion exchangers, AE transcripts from human ciliary body were examined by RT-PCR amplification.

A 1% agarose separation gel was run as shown in Figure 19, and stained with ethidium bromide. The RNA produced cDNAs were from: RNA from human ciliary body (lanes 1 and 2); water control (lane 3), human heart (lane 4) and 293 human embryonic kidney cells (lane 5). The expected migration positions are shown as follows: AE1, 754 bp; AE2, 368 bp; cAE3 982 bp; and bAE3, 891 bp. The cDNA loads derive from the following equivalent amounts of reverse transcribed total RNA: 12.5 ng and 17.5 ng for all lanes 1 and 2; 50 ng for AE1 lanes 4 and 5; 10 ng for AE2, cAE3, and bAE3 lanes 4 and 5.

As illustrated by Figure 19, only AE2 mRNA was expressed in the ciliary body, whereas AE1, cAE3, and bAE3 transcripts were undetectable. 293 cells served as a positive control for AE2 mRNA, and heart served as a positive control for bAE3 and cAE3 mRNAs.

Immunocytochemical detection of AE2 polypeptide. Figure 20A shows bovine PE cells immunostained with flourescent-labeled antibody to the conserved C-terminal amino acids 1224-1237 of mouse AE2 C-terminal peptide in presence of 24 μg/ml irrelevant peptide in accordance with the method described by Alper *et al.*, 1997, 1999, and viewed by indirect immunofluorescence. The mouse peptide differs from the bovine AE2-C by only three residues. The results were identical in the absence of the extraneous peptide.

The staining pattern in the very flat PE cells was consistent with a component of surface membrane localization, with an additional concentration of epitope in a Golgi-like distribution, as described previously by Alper *et al.*, 1997. As shown in Figure 20B, this staining was abolished by the addition of AE2 peptide antigen

(Figure 20B), but the immunostaining was nearly completely retained in the presence of excess of the corresponding AE3 C-terminal peptide antigen (Figure 20C). The abolition of the immunostaining signal by AE2 peptide antigen, while the staining is nearly completely retained in the presence of the corresponding AE3 peptide antigen significantly supports the specificity of AE2 immunostaining in the PE cells. By comparison, no specific immunostaining was detected with affinity-purified polyclonal anti-peptide antibodies to mouse AE1, or to human AE3.

In sum, therefore, it is clear in light of the present invention that paired NHE-1 Na^+/H^+ and AE2 Cl^-/HCO_3^- antiports are important regulatory components in the initial step in aqueous humor formation. Thus, reduction of the H^+ and HCO_3^- to the antiports inhibits the initial step in aqueous humor secretion, permitting more effective blocking of increased intraocular pressure in patients, including glaucoma patients.

The disclosures of each patent, patent application and publication cited or described in this document are hereby incorporated herein by reference, in their entirety.

While the foregoing specification has been described with regard to certain preferred embodiments, and many details have been set forth for the purpose of illustration, it will be apparent to those skilled in the art without departing from the spirit and scope of the invention, that the invention may be subject to various modifications and additional embodiments, and that certain of the details described herein can be varied considerably without departing from the basic principles of the invention. Such modifications and additional embodiments are also intended to fall within the scope of the appended claims.